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ELECTROCHEMICAL BEHAVIOUR OF PROTEINS AT GRAPHITE ELECTRODES. II. ELECTROOXIDATION OF AMINO ACIDS

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Electrochemical oxidation of L,α-amino acids at a paraffin-wax impregnated spectroscopic graphite electrode (WISGE) was studied by means of linear sweep, cyclic, phase-sensitive alternating current and differential pulse voltammetric techniques. It was found that out of the amino acids usually occurring in proteins only tyrosine, tryptophan, histidine, cystine, cysteine and methionine were oxidized at the WISGE. At relatively low concentrations of amino acids (up to ca. 2×10^{-4} M) the electrode process in which the amino acids are oxidized at the WISGE has the characteristics of an irreversible reaction controlled by diffusion. Coulometric measurements showed that oxidation of tyrosine and tryptophan at the WISGE, i.e. of amino acids which are responsible for the oxidizability of proteins at graphite electrodes, is a two-electron process. At higher concentrations of tyrosine and tryptophan (above ca. 2×10^{-4} M) adsorption of the oxidation product of these amino acids was demonstrated.

1. Introduction

It has been shown in our preceding communications [1,2] that proteins can yield oxidation currents at graphite electrodes. We have found that the occurrence of these currents is conditioned by the accessibility of tyrosine or tryptophan residues in the protein for interaction with the graphite electrode. This new electrochemical activity of proteins thus offers the possibility of using methods of electrochemical analysis in investigations of those proteins which contain neither cystine, nor cysteine, nor an electroactive non-protein group. In order to ensure the most precise interpretation of results obtained by this method it is necessary to have as much data as possible on processes by which proteins are oxidized at graphite electrodes. It is apparent that basic information of this kind could be obtained from studies of the behaviour of the building stones of proteins, i.e. amino acids, at graphite electrodes.

Up to now relatively little attention has been paid to studies of electrooxidation of amino acids, in spite of the fact that there exist several papers describing oxidation of amino acids by chemical agents or by enzymes (see, e.g., refs. [3–5]). Of amino acids, electrochemical oxidizability has been found for cystine and cysteine at a platinum electrode (see, e.g., refs. [6–9]) and at a gold electrode [8,10]. Moreover Hampson et al. [11,12] described electrooxidation of all α-amino acids. They found that all α-amino acids are oxidizable at these electrodes at approximately the same potentials. This oxidation leads to the formation of an imine intermediate, which is further oxidized to nornitril. At a silver electrode slow hydrolysis of this intermediate to noraldehyde also takes place.

Electrochemical oxidizability of six L, α-amino acids at graphite electrodes is demonstrated in the present paper; besides cystine and cysteine, methionine, histidine, tyrosine and tryptophan are also electrochemically active. We also obtained some basic data on processes by which these amino acids are oxidized at graphite electrodes, especially with the aim of contributing to the understanding of the mechanism by which proteins are oxidized at graphite electrodes.

2. Materials and methods

All L, α-amino acids were obtained from Calbiochem. (California). Chemicals used for the preparation of back-

ground electrolyte solutions (all of analytical grade) were obtained from Lachema (Brno). The paraffin-wax impregnated spectroscopic graphite electrodes (WISGE) were made and used in the same way as described earlier [13]. The WISGE No 1 and No 2 had geometric areas of ca. 30 mm^2 and 7 mm^2 respectively. The Metrohm hanging mercury drop electrode (HMDE) E 410 had a surface area of 3.5 mm^2.

Voltammograms of amino acids were obtained in a 2 ml capacity thermostatted cell. A three-electrode system was used, including the WISGE or HMDE, a Pt counter-electrode, and a saturated calomel reference electrode (SCE). Linear sweep (LS), cyclic and phase sensitive alternating current (AC) voltammetric measurements were carried out with a GWP 673 Multimode Polarographic Analyzer (GDR). For AC voltammetry a phase angle of 90° with respect to the applied alternating voltage, a direct current ramp of 0.004 Vs^{-1}, and a modulating voltage of 80 Hz and 0.020 V peak-to-peak were employed in all experiments. Differential pulse (DP) voltammetric measurements were performed with a prototype of a pulse polarograph PA 3 (Laboratory Instruments, Prague). Fast scan DP voltammograms of amino acids were obtained with pulse amplitude of 50 mV and sweep rate of 20.0 mVs^{-1}. The current sampling for DP voltammetry was set with the drop time control of PA 3 at 0.2 s. LS, AC and DP voltammograms were recorded with an Endim 620.02 recorder (GDR). Cyclic voltammograms were recorded with an OG2-21 Speicheroszilloskop, VEB Messelektronik Berlin (GDR) in the X-Y mode.

The procedures for LS, cyclic and DP voltammetry of amino acids at the WISGE were only slightly different from those used by us for voltammetry of DNA [13,14]. Once the WISGE was inserted into the tested solution contained in an electrochemical cell, it was allowed to stand for 10 s without applied potential. Then initial potential of 0.0 V or 0.2 V was applied for the next 60 s, after which time the voltammetric sweep was commenced. In some experiments a magnetic stirrer rotated at a speed of ca. 300 r.p.m. at the bottom of the electrochemical cell during the first 30 s with initial potential applied; stirring the solutions in the described manner did not, however, influence the course of the voltammograms. Techniques outlined in detail elsewhere [15] were employed to obtain reproducible data by phase-selective AC voltammetry at the WISGE. The macroscale electrolysis at controlled potential and coulometry were performed with the aid of a GWP 673 three-electrode system. The working electrode was represented by a bundle of seven WISGE's, each of them having a geometric area of 30 mm^2. We electrolyzed 8 ml of tyrosine or tryptophan solution at a concentration of 2×10^{-4} M in 0.2 M acetate buffer or Britton–Robinson buffer. A magnetic stirrer rotated at the bottom of the cell at a speed of 300 r.p.m. during electrolysis. All electrochemical measurements were carried out with the voltammetric cell maintained at 25 °C. The potentials reported in this paper are given against the SCE at 25°C.

Ultraviolet (UV) spectra were measured with a Unicam SP 700 recording spectrophotometer.

Other details of our measurements were published elsewhere [13,14].

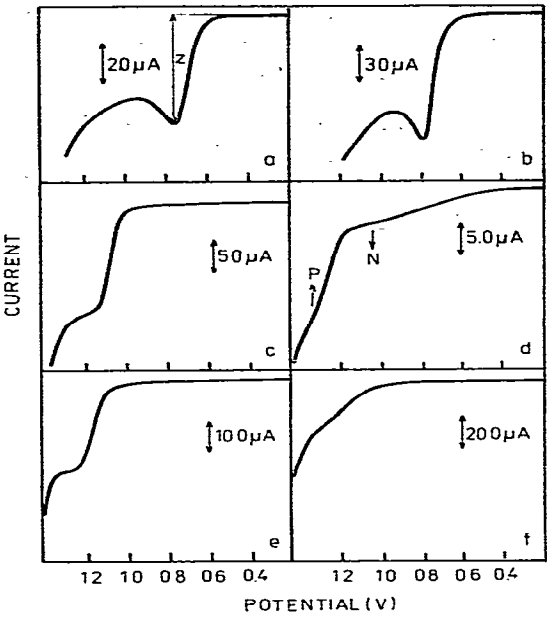


Fig. 1. Linear sweep voltammograms of (a) 1×10^{-4} M tyrosine, (b) 1×10^{-4} M tryptophan, (c) 1×10^{-4} M histidine, (d) 1×10^{-4} cysteine, (e) 1×10^{-4} M cystine, (f) 2×10^{-4} M methionine in Britton – Robinson buffer, pH 7.4. Voltage scan rate 16.66 mVs^{-1}, initial potential 0.2 V, WISGE No. 1.

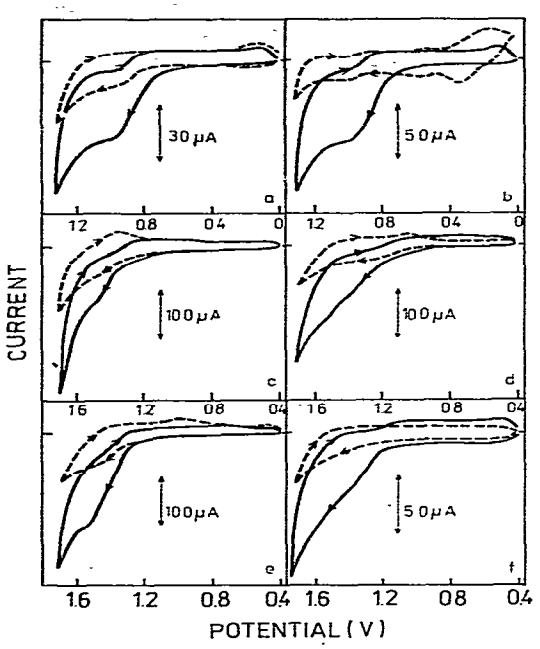


Fig. 2. Cyclic voltammograms of amino acids at the WISGE in Britton–Robinson buffer, pH 4.0. (a) 1×10^{-4} M tyrosine, (b) 1×10^{-4} M tryptophan, (c) 1×10^{-4} M histidine, (d) 1×10^{-4} M cysteine, (e) $(1 \times 10^{-4}$ cystine, (f) 1×10^{-4} methionine. (——) first scan at a clean electrode, sweep started at 0.0 V (a, b) and at 0.4 V (c–f), and run toward positive potentials; (– – –) steady-state voltammograms. Calibration marks on current axis indicate zero current for the appropriate voltammograms. Current below the calibration mark is anodic. Voltage scan rate 1 Vs^{-1}, WISGE No. 2.

3. Results and discussion

3.1. Oxidizability of amino acids at graphite electrodes

LS voltammograms at the WISGE for all amino acids commonly occurring in proteins were recorded in the medium of Britton–Robinson buffer, in the range of pH 2.5–10.5. We found out that only tyrosine, tryptophan, histidine, cystine, cysteine and methionine yielded peaks on voltammograms. Typical LS voltammograms at the WISGE of these amino acids at pH 7.4 are shown in fig. 1. No other peaks were observed on LS voltammograms of amino acids, even if the initial potential was 0.0 V.

Our further study was directed to obtaining some information on the nature of the process responsible for the formation of the described voltammetric peaks of amino acids. First we recorded cyclic voltammograms for all six amino acids yielding voltammetric peaks. In the range of voltage scan rates (v) 0.01–25 Vs^{-1} cyclic voltammograms had qualitatively identical courses. Cyclic voltammograms at $v = 1$ Vs^{-1} are shown in fig. 2. Peak potentials were shifted with increasing scan rate and amino acid concentration to more positive values, and no amino acids, with the exception of tryptophan, yielded, any cathodic counter-peak. Tryptophan, however, yielded this counter-peak. The results of cyclic voltammetry thus suggest that tyrosine, tryptophan, histidine, methionine, cystine and cysteine are oxidized at the WISGE in an irreversible process.

The theoretical equation for a linear, diffusion-controlled, irreversible-peak voltammogram at a planar electrode is [16]:

$$(i_p)_{irrev} = 2.98 \times 10^5 n\,(\alpha n_a)^{1/2} A D^{1/2} C\,. \qquad (1)$$

where $(i_p)_{irrev}$ is the peak current in μA, n the total electron number, α the electron transfer coefficient, n_a the number of electrons involved in the rate-controlling electron transfer process, A the electrode area (cm^2), D the diffusion coefficient of the electroactive species ($cm^2 s^{-1}$), v the voltage scan rate (Vs^{-1}), and C the bulk concentration of the electroactive species (mM). According to this equation, for processes controlled by diffusion i_p should increase linearly with C and the dependence $\log i_p$ versus $\log v$ should be a line with the slope close to 0.5. Peak currents of all six amino acids oxidizable at the WISGE satisfied the two latter criteria in the range of relatively low amino acid concentrations (up to ca. 2×10^{-4} M). It can be concluded that the oxidation at the WISGE of amino acids in relatively low concentrations has the characteristics of a diffusion controlled process.

Concentration limits for the determination of amino acids oxidizable at graphite electrodes by means of LS voltammetry at the WISGE are at about 1×10^{-6} M for tyrosine and tryptophan, at about 3×10^{-5} M for histidine and methionine and at about 3×10^{-6} M for cystine and cysteine. These limits can be somewhat decreased (but by no more than one order of magnitude) by using the more sensitive differential pulse voltammetry. If a microcell with a volume of several tenths of a ml is employed, nanogram amounts of tyrosine and

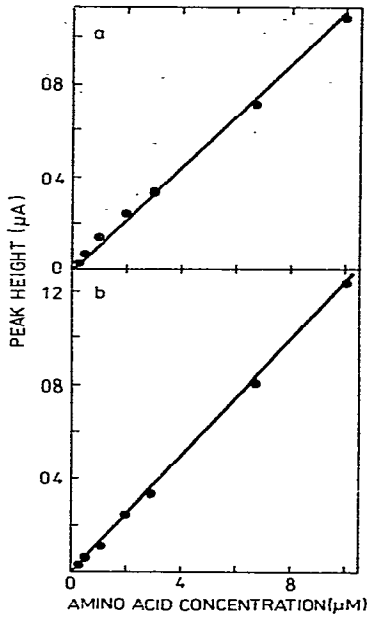


Fig. 3. Variation of the differential pulse voltammetric peak height with tyrosine (a) and tryptophan (b) concentration in 0.2 M sodium acetate, pH 6.5. Unbroken lines represent the dependences obtained for tyrosine (a) and tryptophan (b) solutions to which no other amino acid was added. Solid circles represent the experimental points obtained for tyrosine (a) and tryptophan (b) in the presence of the mixture of other amino acids usually occuring in proteins, all at a concentration of 5×10^{-5} M.

tryptophan can thus be determined even in mixtures with other amino acids (fig. 3).

Dependence of the voltammetric peak heights and of the peak potentials on pH for amino acids oxidizable at the WISGE are shown in figs. 4 and 5 respectively. Fig. 4 demonstrates that the heights of amino acid peaks are strongly dependent on pH. The peak potentials of all oxidizable amino acids are shifted to more positive values with decreasing pH (fig. 5). With the exception of the more negative peak of cysteine, E_p was shifted linearly with changing pH; a break in the vicinity of pH corresponding approximately to the isoelectric point of the particular amino acid could be observed on these linear pH-dependences for methionine and cystine. The shifts of the peak potential per pH unit of 58 mV for tyrosine, 51 mV for tryptophan, and 62 mV for histidine are in fairly close accord with

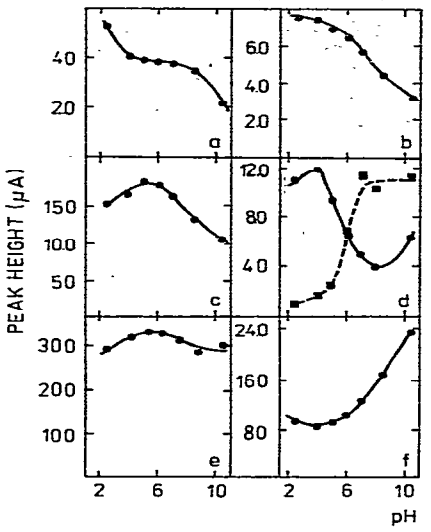


Fig. 4. Variation of the linear sweep voltammetric peak height with pH for amino acids at a concentration of 1×10^{-4} M in Britton–Robinson buffer. (a) tyrosine; (b) tryptophan; (c) histidine; (d) cysteine: (•) more negative peak, (▪) more positive peak; (e) cystine; (f) methionine. Initial potential 0.0 V, voltage scan rate 8.33 mVs^{-1} (a, b) and 16.66 mVs^{-1} (c–f), WISGE No. 1.

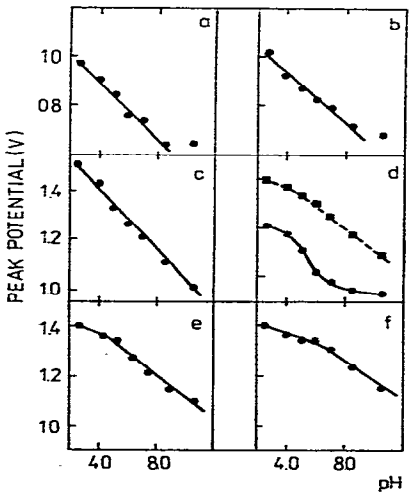


Fig. 5. Variation of the linear sweep voltammetric peak potential with pH for (a) tyrosine, (b) tryptophan, (c) histidine, (d) cysteine, (e) cystine, (f) methionine. Other conditions as in fig. 4.

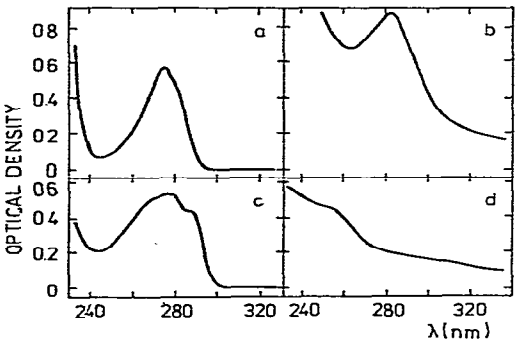


Fig. 6. Ultraviolet absorption spectra of 2×10^{-4} M tyrosine, 2×10^{-4} M tryptophan, and of products of their macroscale electrolysis at the WISGE's in 0.2 M sodium acetate, pH 4.0. (a) tyrosine, (b) product of tyrosine electrolysis, (c) tryptophan, (d) product of tryptophan electrolysis. (a, b) 2 cm light-path cell, (c,d) 0.5 cm light-path cell.

those expected for a process where the rate-controlling step involves identical numbers of protons and electrons.

3.2. Characteristics of electrode reactions of tyrosine and tryptophan at a graphite electrode

Some further data, concerning electrode processes in which amino acids are oxidized at the WISGE, will be given in this study only for tyrosine and tryptophan, i.e. for amino acids whose oxidation at the WISGE has been proved even in proteins [1,2]. Further information on processes in which other amino acids are oxidized at graphite electrodes will be published elsewhere [17].

An important characteristic of irreversible electrode processes is the value αn_a defined by equation (1). Several methods allowing its estimation have been described [18]. In the present study we calculated αn_a by exploiting a modified equation derived by Nicholson and Shain [16], which is valid for potentials corresponding to the foot of the voltammetric peak:

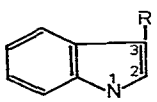
$$n_a = \ln(i_1/i_2) \times RT/F(E_1 - E_2), \qquad (2)$$

where i_1 and i_2 are values of currents at potentials E_1 and E_2, respectively, R is the gas constant, T absolute temperature, and F the Faraday charge. For the calculation of αn_a according to the equation (2) we used values i and E taken from voltammograms obtained for tyrosine and tryptophan at a concentration of 1×10^{-4} M and voltage scan rate of 0.016 Vs^{-1}. Values of αn_a ranging from 0.95 to 1.08 and from 0.74 to 0.84 were obtained for tyrosine and tryptophan respectively, at pH 2.5–8.6. Taking a reasonable value of $\alpha = 0.5$, it can be concluded that the number of electrons involved in the rate-controlling electron transfer process, n_a, is 2 for both tyrosine and tryptophan.

At pH 3–8, coulometry at potentials corresponding to the crests of the tyrosine and tryptophan oxidation peaks gave faradaic n values ranging from 1.9 to 2.2. At the completion of electrolysis, characteristic UV absorption spectra were recorded (fig. 6).

The course of the UV absorption spectrum of the product of electrooxidation of tyrosine enables us to conclude that probably no significant destruction of the aromatic nucleus of tyrosine takes place during its electrooxidation at the WISGE. Voltammetric inactivity of phenylalanine at the WISGE demonstrates the important role of the hydroxyl group attached to the benzene ring of tyrosine for its oxidizability at the WISGE. Moreover, analyses of tyrosine by means of fast cyclic voltammetry at the WISGE and of the product of tyrosine electrolysis by means of polarography and of fast cyclic voltammetry at the HMDE and the WISGE did not prove the formation of a substance of quinoid character either as an intermediate product or as the final product of the electrode reaction. We therefore suppose that 3,4-dihydroxyphenylalanine could probably be the primary product of the two-electron oxidation of tyrosine. This substance is formed by tyrosine oxidation in the presence of phenoloxidase [5].

Cyclic voltammetry (fig. 2) showed that the electrooxidation of tryptophan at the WISGE is accompanied by follow-up chemical reactions [18]. The decrease of absorption maximum of tryptophan at 278 nm in the course of the electrolysis indicates that partial destruction of the indole nucleus takes place during tryptophan oxidation at the WISGE. It is a well-established fact that the benzene ring of indoles is less reactive than the pyrrole ring, and that substitution reactions lead to indole derivatives substituted exclusively in position 3. Accordingly, it is possible to suggest that oxidation of the double bond



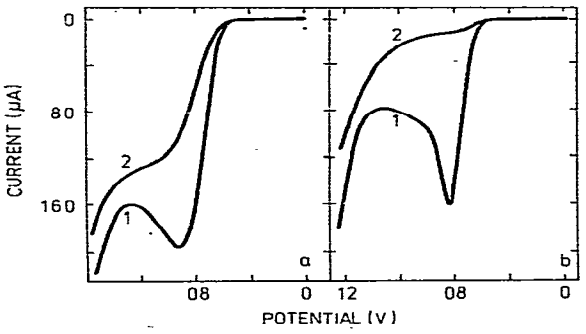


Fig. 7. Linear sweep voltammograms in 0.2 M sodium acetate, pH 6.5, of (a) 2.5×10^{-3} M tyrosine, (b) 5×10^{-3} M tryptophan. Curves 1 represent the first voltammetric traces at a clean, resurfaced WISGE. Curves 2 represent the second voltammetric traces obtained at the same WISGE used to obtain curves 1 without resurfacing the electrode. Voltage scan rate 20.0 mVs^{-1}, initial potential 0.0 V, WISGE No 2.

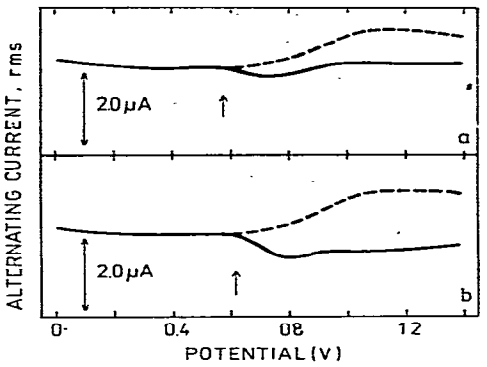


Fig. 8. Alternating current (AC) voltammograms in 0.2 M sodium acetate, pH 6.5, of (a) 1.25×10^{-3} M tyrosine, (b) 5×10^{-3} M tryptophan. The dashed line is the curve of pure supporting electrolyte, the unbroken line for the background electrolyte plus the organic sample. A phase angle of 90° with respect to the applied alternating voltage was employed. The arrows indicate the potential of the foot of the LS voltammetric oxidation peak, which was recorded under the same conditions as AC voltammograms. Initial potential 0.0 V, WISGE No. 2.

–C(2) = C(3)– is probably the primary reaction of the two-electron process by which tryptophan is oxidized at the WISGE. Indole ring of tryptophan is changed in the same way, e.g. upon its oxidation with molecular oxygen in the presence of tryptophan oxidase [5].

The preceding conclusions on the electrode process, during which tyrosine and tryptophan are oxidized at the WISGE concern only those experimental conditions in which concentration of these amino acids was relatively low (up to ca. 2×10^{-4} M). At higher concentrations the peak current ceased to increase linearly with the increasing amino acid concentration; at concentrations higher than ca. 2×10^{-3} M the peak current tended to a limiting value. These results indicate that at higher concentrations of tyrosine and tryptophan one of the forms of these amino acids is adsorbed at the WISGE [19]. Therefore we endeavoured to find out which form of these amino acids is adsorbed at the WISGE under these conditions. The first traces in fig. 7 (curves 1) represent LS voltammograms of solutions of 2.5×10^{-3} M tyrosine and 5×10^{-3} M tryptophan in 0.2 M sodium acetate, pH 6.5, at a clean, freshly resurfaced electrode. The second traces (curves 2 in fig. 7) were obtained under the same conditions as the first traces, except that the WISGE was not resurfaced. It can be seen from curves 2 (figs. 7a, b) that the oxidation currents of both tyrosine and tryptophan are substantially lowered. This clearly suggests that the products of electrochemical oxidation of tyrosine and tryptophan are strongly adsorbed and largely block the electrode surface to fresh amino acid molecules.

Adsorption of tyrosine and tryptophan (at relatively high concentrations) could also be demonstrated by immersing a WISGE in a solution of 2.5 mM tyrosine or tryptophan, followed by application of a potential of 0.2 V for 120 s. After this time the electrode was removed from the solution and washed with an appropriate solution of acetate buffer. After 30 s the WISGE was inserted in an identical electrochemical cell which contained only pure acetate buffer solution. A potential of 0.2 V was again applied for 120 s, and then a LS voltammogram was recorded. No voltammetric peak was formed. The same result was obtained when initial potential in the range of 0.0–0.7 V was applied. This indicates that the nonoxidized forms of tyrosine and tryptophan were not strongly adsorbed at the WISGE even at concentrations corresponding to almost saturated amino acid solutions.

The adsorption of organic substances at graphite electrodes can also be demonstrated by means of phase-sensitive AC voltammetry. In the case of the adsorption of an organic substance at the graphite electrode, the quadrature current (which may be regarded as being approximately proportional to the dif-

ferential capacitance of the electrode/solution interface [20]) is reduced compared to that for pure supporting electrolyte. In this way, e.g., adsorption of purine bases occurring in nucleic acids and of their nucleosides and nucleotides at a graphite electrode have been demonstrated [14,15].

In accord with our preceding conclusions no significant adsorption of nonoxidized forms of tyrosine and tryptophan at the WISGE was observed, which could be reflected in the lowering of the quadrature current (fig. 8). Only at potentials more positive than the oxidation potentials of tyrosine and tryptophan one could observe a marked decrease of the qaudrature current (fig. 8). However, the adsorption of no form of these amino acids was observed by means of the methods used in the present study at amino acid concentrations lower than ca. 1×10^{-4} M. It can thus be summarized than only oxidation products of tyrosine and tryptophan are adsorbed at the WISGE and probably only at high concentrations of these mino acids in solution. This behaviour could be connected with the fact that at high concentrations of tyrosine and tryptophan in solution their oxidation products form an adsorption film at the WISGE as has been suggested by Reynaud and Malfoy [21].

4. Conclusions

Oxidation of tyrosine, tryptophan, histidine, methionine, cystine and cysteine at graphite electrodes, described in this work, represents a new manifestation of of the electrochemical activity of these biologically important substances. Moreover, voltammetric analysis at graphite electrodes offers the possibility of rapid and sensitive determination of tyrosine and tryptophan even in mixtures with other amino acids (e.g. after protein hydrolysis), without separation of amino acids and without addition of other substances to the analyzed solution. The results presented in this work also represent a basis for understanding the electrode process by which proteins are oxidized at graphite electrodes [1, 2].

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